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(21) International Application Number: PCT/GB91/01182 (22) International Filing Date: 17 July 1991 (17.07.91) (30) Priority data: 9016012.8 20 July 1990 (20.07.90) GB (71) Applicant (for CA GB only): UNILEVER PLC [GB/GB]; Unilever House, Blackfriars, London EC4P 4BQ (GB). (71) Applicant (for all designated States except CA GB US): UNILEVER NV [NL/NL]; Burgemeester 's Jacobplein 1, NL-Rotterdam (NL). (72) Inventor; and (75) Inventor/Applicant (for US only): MAWSON, Rodney [GB/GB]; Fairlawn, 135 Huntingdon Road, Thrapston, Northants NN14 4NG (GB).		(74) Agent: BUTLER, David, John; Patent Division, Unilever plc, Unilever House, Blackfriars, London EC4P 4BQ (GB). (81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent), US. Published <i>With international search report.</i>
(54) Title: PIGMENTS (57) Abstract A novel strain of <i>Adonis aestivalis</i> having an average of 18 - 22 petals per flower head and containing an average of 200 - 350 µg of astaxanthin pigment per flower head is cultivated, harvested and extracted to provide a source of natural astaxanthin. The extracted astaxanthin, or the harvested astaxanthin-containing plant material, can be used for example in salmonid fish diets to promote correct flesh pigmentation of the fish.		

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⁺ It is not yet known for which States of the former Soviet Union any designation of the Soviet Union has effect.

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PIGMENTS

This invention relates to carotenoid pigments extractable from natural sources.

There is a need for a cost-effective natural source of astaxanthin, which is the carotenoid pigment that contributes the characteristic pink or red colour to the flesh of wild salmon. Salmonid fish are extensively farmed today, and there is a commercial need to produce such fish possessing a nature-identical flesh colour.

Astaxanthin occurs in various marine animals, such as crustacea, but its extraction from such sources is uneconomic. It is also possible to synthesise astaxanthin, but this is expensive, and moreover the use of such synthetic pigment does not convey the connotation of 'natural' that many consumers regard as desirable.

Astaxanthin occurs in certain plants, especially certain species belonging to the genus Adonis. One such species is Adonis aestivalis, where it occurs predominantly in the petals of the bright red flowers. However, the reported wild strains of Adonis aestivalis

possess only flower heads with very few petals, and the proportion of astaxanthin pigment relative to the total mass of the plant is too small for it to be cultivated and extracted on any sensible commercial scale.

By the invention we have discovered a novel true-breeding strain of Adonis aestivalis having substantially heavier flower heads, and in particular having a substantially greater number of petals. The proportion of astaxanthin pigment in the plant is sufficiently high to make cultivation of the plant for the purposes of extracting the pigment commercially attractive.

The invention provides plants of the genus Adonis having petals containing astaxanthin, the average number of petals per flower head being at least 10, more particularly at least 16.

The invention also provides plants of the genus Adonis having petals containing astaxanthin, wherein the amount of astaxanthin per flower head is at least 100 μ g, more particularly at least 150 μ g, and yet more particularly at least 200 μ g.

The invention includes the cultivation of such plants for the purpose of obtaining astaxanthin, the extraction of astaxanthin from such plants, and the astaxanthin so obtained.

Extraction of the astaxanthin is preferably conducted using an organic solvent, and more preferably using a mixed solvent comprising a water-miscible organic solvent (such as ethanol) and a non-water-miscible organic solvent (such as hexane).

Preferably, the harvested plant material is initially extracted with water or aqueous media to remove water soluble compounds such as glycosides.

The invention particularly provides a process for obtaining astaxanthin, wherein plants of the species Adonis aestivalis having an average flower head petal number of at least 16 are cultivated, harvested, and the astaxanthin is extracted from the harvested flower heads or petals thereof.

An important embodiment of the invention is a newly-discovered strain of Adonis aestivalis of which a seed sample has been deposited on 18 July 1990 with the National Collection of Industrial and Marine Bacteria Limited, Aberdeen, under Accession No. NCIMB 40309, in accordance with the provisions of the Budapest Treaty. The invention encompasses plants of the species Adonis aestivalis having the essential characteristics of this deposited strain. Plants of this deposited strain typically have an average of 18-22 petals per flower head, and the average amount of astaxanthin per flower head is 200-350 μ g.

The invention also provides a process for obtaining astaxanthin, wherein the astaxanthin is extracted from the petals of plants having the essential characteristics of the deposited strain.

The invention particularly provides an oral composition for administration to fish, comprising such extracted astaxanthin, and a method of pigmenting the flesh of fish, especially salmonid fish, involving the oral administration to the fish of such a composition.

Preferably, the composition comprises the astaxanthin mixed with edible feed material. Alternatively, the astaxanthin can be in encapsulated form.

Alternatively, pigmentation of the flesh of fish can be achieved by feeding astaxanthin-containing portions of the plant to the fish. Preferably, the portion comprises the flower petals and more preferably, consists entirely of such material. If desired, the plant material can be extracted with water or aqueous media in order to remove water-soluble compounds such as glycosides which may be toxic to fish or other animals, without removing significant quantities of the required astaxanthin.

In the pigmentation of farmed fish, the astaxanthin obtained by the invention can be administered orally to the fish in any manner analogous to the techniques already used for astaxanthin derived from conventional sources. Normally the pigment is included in a composition, such as a pelleted compound feedstuff, that forms all or part of the diet on which the fish are reared. The pigment is soluble in oil, and can be incorporated in the diet in this form, either as free oil or as encapsulated oil. Alternatively, the petals or other plant material containing the astaxanthin can be mixed (eg. in dried, ground form) with conventional fish feed ingredients. If desired, the plant material can be partially extracted with aqueous media (to remove water-soluble components such as glycosides) prior to being added to the feed. As a further alternative, the pigment can be added to the feed in the form of an organic solution, eg. a solution obtained during extraction of the astaxanthin from the plant material, if the organic solvent used is not toxic to the fish in any

amount that the fish are likely to ingest via the completed feedstuff.

When the astaxanthin is administered to fish via their feedstuff, the composition of the feedstuff need not be unconventional. The feedstuff formulation can contain any of the normal fish feed components, such as fish meal and/or other protein, oil such as fish oil, cereals, vitamins, minerals, preservatives and medicaments, in the various proportions that are normally used.

The extracted astaxanthin, or astaxanthin-containing portions of the plant, can also be used as a colouring agent in human foodstuffs, and also in poultry diets to enhance the colour of egg yolks.

Specific embodiments of the invention will now be described in detail, by way of example only.

Novel strain of *Adonis aestivalis*

The essential characteristics of the newly-discovered strain are:

Chromosome No. $2n = 32$

Erect, annual, leaves alternative, pinnately dissected into linear or filiform segments. Flowers bright red, actinomorphic, hypogynous, hermaphrodite, solitary and terminal on stem and branches.

Petals less than 15mm long, up to 2 times length of sepals. Sepals 5-8 in number. Petals 18-22 in number.

Values for these parameters, and for the astaxanthin level per flower head, can be taken from analysis of the first fully-open flower heads from 1000 plants.

Ripe achenes 3-5mm, tooth on dorsal surface distant from the beak. Achenes having a transverse ridge passing around middle of achene. Achenes also having a dorsal hump a distance from the beak.

Cultivation

The plant can be grown under a wide range of temperature conditions. Germination requires a degree of alternating temperatures of 10-20°C. Mature flowering plants are obtained four months after sowing at field densities up to 150 plants/m² in a variety of soil conditions; flowering in the summer months (temperate climate). The plant prefers dry, well drained conditions. Seed can be harvested by combine, and flowers by hand.

Extraction of pigment

Pigment can be extracted by solvent extraction, eg. into mixed solvents such as ethanol/hexane, and further purified by partitioning a mixed solvent with water followed by column chromatography. The final extract, thus purified, is rich with respect to carotenoids, and predominant is astaxanthin, present mainly as a racemic mixture in the form of mono- and diesters, generally of palmitic acid.

Typical starting material

Frozen block of flower heads, stored at -20°C in the dark, or dried petals/flowerheads, finely ground, also stored at low temperature in the dark.

Primary Extraction

Mix with 10 vols hexane/ethanol (50:50 v/v), allow dispersion, and homogenise for 10 minutes in dark and cool conditions using shear blender; or mill with a shear blender in ethanol, then add hexane for safety reasons.

Leave overnight in dark and cool conditions.

Filter through filter (eg. muslin) on vibrating sieve, and wash plug with 50:50 ethanol:hexane. Retain original filtered liquid and washings as primary extract. This primary extract contains water-soluble, ethanol-soluble and hexane-soluble material including pigments and glycoside.

Secondary Extraction

Add 1 part water to 2.5 parts primary extract, transfer to phase separator and remove bottom layer of ethanol. Wash upper hexane layer with 1:1 ethanol/water mixture allow to separate and discard lower layer.

Transfer to steam jacketed vacuum evaporator (with cyclone) and remove hexane at 45°C for 15-30 minutes until a sludge is obtained. Wash with ethanol and evaporate, wash with hexane and evaporate (again at low temperatures, under vacuum or under nitrogen) to dryness. This yields a first concentrate of approximately 5% total pigment (80% astaxanthin) in dry matter.

Take up in hexane and apply to silica column (1:1 or 2:1 extract to silica). Wash column with hexane in dark and cool conditions, and discard washings. Elute with 2.5% ethanol in hexane until a red-orange band appears. Collect the red-orange washings until the colour changes to orange-green.

Dry eluent as before, take up in hexane or oil (fish oil, vegetable oil). This yields a second concentrate of approximately 20% total pigment containing approximately 80% astaxanthin.

Typical Salmon Grower Diet

	<u>% inclusion by weight</u>
Fishmeal	75.0
Vegetable protein	5.0
Cereal	7.8
Fish Oil	11.0
Minerals/Vitamins	1.0
Antioxidants/preservatives	0.2

Pigment incorporation

Astaxanthin pigment from an Adonis strain of the invention can be added at levels ranging for example from 1-100 ppm to the above type of diet in a variety of ways:

- a) as extracted astaxanthin carried in a fish oil base, optionally containing antioxidants.
- b) as extracted astaxanthin carried as a free-flowing powder in wheatflour or any finely ground foodstuff for salmon.

- c) as for (b) but encapsulated in alginate, gelatin or xanthan gum, eg. by pan granulation or spray cooling.
- d) as extracted astaxanthin carried in an encapsulated lipid, eg. casein-protected lipid.

Pigmentation

The above typical salmon grower formulation containing 50-100 ppm of astaxanthin extracted from the deposited Adonis strain of the invention and carried in one of the product forms described above, is fed as pellets to fish of 300 g liveweight plus for a minimum period of 3 months. Typical values for pigmentation efficiency, compared with commercially-available synthetic astaxanthin, are at least about 80% of synthetic.

The skilled reader will readily appreciate that the foregoing extraction procedures, diet formulations, feeding regimes and other details may be subject to considerable variation without departing from the spirit of the invention.

CLAIMS

1. A process for the production of astaxanthin wherein the astaxanthin is extracted from plants of the genus Adonis having an average of at least 10 petals per flower head.

2. A process according to claim 1, wherein the plants have an average of at least 16 petals per flower head.

3. A process for the production of astaxanthin wherein the astaxanthin is extracted from plants of the species Adonis aestivalis having an average of 18 to 22 petals per flower head.

4. A process for the production of astaxanthin wherein the astaxanthin is extracted from plants of the strain NCIMB 40309.

5. A process according to any one of the preceding claims, involving cultivation of the plants, harvesting of the flower heads and extraction of the astaxanthin from the harvested flower heads or petals thereof.

6. A process according to any one of the preceding claims, wherein the extraction of the astaxanthin is conducted using an organic solvent.

7. A process according to claim 6 wherein the extraction is conducted using a mixed solvent comprising a water-miscible organic solvent and a non-water-miscible organic solvent.

8. A process according to claim 6 or claim 7, wherein the harvested plant material is initially extracted with

water or aqueous media to remove water soluble compounds such as glycosides.

9. Astaxanthin produced by a process according to any one of the preceding claims.

10. Plants of the genus Adonis having an average of at least 10 petals per flower head, and containing per flower head at least 100 μg of astaxanthin.

11. Plants of the genus Adonis having an average of at least 16 petals per flower head, and containing per flower head an average of at least 100 μg of astaxanthin.

12. Plants of the species Adonis aestivalis having an average of 18 to 22 petals per flower head, and an average amount of astaxanthin per flower head of 200 to 350 μg .

13. Plants of strain NCIMB 40309.

14. A composition for use in the pigmentation of fish, comprising astaxanthin produced by a process according to any one of claims 1 to 8 mixed with edible feed material.

15. A composition for use in the pigmentation of fish, comprising astaxanthin produced by a process according to any one of claims 1 to 8 in encapsulated form.

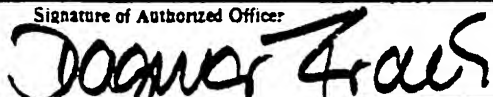
16. A process for the pigmentation of fish, involving oral administration of a composition according to claim 14 or claim 15.

17. A feedstuff for fish incorporating harvested material from plants according to any one of claims 10 to 13.

18. Use of plants according to any one of claims 10 to 13 as a source of "natural" astaxanthin.

19. The cultivation of plants according to any one of claims 10 to 13 for the purpose of astaxanthin production.

20. Harvested flower heads or petals of plants according to any one of claims 10 to 13.

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC Int. Cl. 5 B 61/00 A 23 L 1/27		
II. FIELDS SEARCHED		
Minimum Documentation Searched:		
Classification System	Classification Symbols	
Int. Cl. 5	C 09 B A 23 L	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	Biochemical Systematics and Ecology, vol. 9, no. 4, 14 February 1981, Pergamon Press Ltd, (GB), B. Renstrom et al.: "Esterified, optically pure (3S,3'S)-Astaxanthin from flowers of Adonis annua", pages 249-250, see pages 249-250 ---	1-9, 18, 19
X	Nature, vol. 184, 28 November 1959, (London, GB), A. Seybold et al.: "Occurrence of astaxanthin in the flower petals of Adonis annua L.", pages 1714-1715, see pages 1714-1715 ---	1-9, 18, 19
A	WO, A, 9005765 (E. MIKALSEN) 31 May 1990, see page 1, lines 1-13; page 5, lines 15-25; claim 1 -----	1-9, 14-19
<p>⁹ Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family.</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report.	
18-09-1991	30. 10. 91	
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer: 	

V. ☒ OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim numbers 10-13;20 because they relate to subject matter not required to be searched by this Authority, namely:

Please see PCT-RULE 39.1(ii):

Plant or animal varieties or essentially biological processes for the production of plants or animals, other than microbiological processes and the products of such processes.

2. ☐ Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the International application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

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This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 23/10/91. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 9005765	31-05-90	AU-A- 4637789	12-06-90
		EP-A- 0444103	04-09-91